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TITLE: Inhalation of Uranium Oxide Aerosols: CNS Deposition, Neurotoxicity, and Role in Gulf War Illness

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14. ABSTRACT

This study investigates the potential for inhaled uranium oxide (UO) aerosols to penetrate the nose-brain barrier, directly enter the central nervous system (CNS), distribute within the CNS, and result in slowly developing neurotoxicity. Inhalation exposures to depleted uranium (DU) may have occurred during the GW in several scenarios of varying duration and airborne uranium concentration. Nasal inflammation could alter sensitivity to uranium uptake. Nephrotoxic and pulmonary effects are evaluated to determine whether CNS effects can occur at lower thresholds than nephrotoxic effects. In year 4, we focused on analysis of tissues following long-term (30 days), moderate dose (1 mg/m3) uranium inhalation, with or without induced nasal inflammation. Brain uptake was only seen in a subset of rats (2 of 12) and only in the olfactory bulb. Similarly, rats re-exposed to long-term, low dose uranium in combination with induced nasal damage also showed a limited uranium uptake (3 of 24 rats). Histological evaluation of olfactory bulbs revealed an increased astrogliosis and an upregulation of tyrosine hydroxylase (a marker for dopaminergic neurons) 180 days after uranium inhalation. No loss of large spinal motor neurons was seen at the same timepoint. Uranium-associated kidney pathology was not notable at this exposure regimen, but alveolar macrophage hyperplasia and particle inclusion were uniform and persistent, apparent even at 180 days post 30 day exposure.

15. SUBJECT TERMS
Uranium, depleted uranium, nose-brain barrier, Gulf War Disease, neurotoxicity, toxicity

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INTRODUCTION

Purpose: The purpose of the overall project is to test the hypothesis that inhaled uranium–containing aerosols will enter the central nervous system (CNS) via olfactory transport, follow neuronal pathways to distal regions of the CNS, and ultimately result in neurodegeneration. The studies in Year 4 address metal uptake and neuronal damage after long term, moderate concentration exposures to uranium oxides. These studies examine the effects of inhaled uranium oxides both in a healthy rat model and one in which inflammation has been induced in the upper respiratory tract. Brain uranium uptake is measured immediately after exposure. Histological markers of neuronal damage and inflammation in brain and spinal cord is measured immediately after and 6 months after exposure. Other target organs to be examined include nasal tissue, lungs and kidneys. Results of these exposures will address all of the original hypotheses:

Hypothesis I. Inhalation of uranium aerosols during the Gulf War from combustion of DU containing weapons resulted in CNS deposition and subsequent neurodegeneration in a subset of those exposed.

Hypothesis II: Transient conditions such as inflammation compromised the olfactory epithelium and enhanced the entry of uranium and the subsequent development of neurodegeneration

Hypothesis III: Markers of neurodegeneration are correlated with the concentration and deposition of U within the CNS following inhalation exposure.

Hypothesis IV: The degree of and time-course of neurodegeneration are dose and exposure duration dependent.

These hypotheses will be tested for both short- and long-term exposure scenarios as differences related to exposure rate and dose may be critical to uptake, clearance, and ultimate neurotoxicity. The following inhalation ***exposure scenarios*** are used:

–***Tank-Impact Scenario:***
Acute (15 min) – high-level concentrations (500 mg/m^3)

–***March-Through Scenario:***
Short duration (6 h) – moderate concentration (1 mg/m^3)

–***Clean-Up Scenario:***
Longer duration (6h/day, 5 days/week for 30 days) – moderate concentration (1 mg/m^3)

–***Re-exposure Scenario:***
Long duration (6h/day, 5 days/week for 30 days) - moderate concentration (1mg/m^3) 11 months after initial long or short duration – moderate concentration

BODY

Approved Scope of Work for Year 4

Year 1-3 Scope as a prelude to Year 4 tasks

During Year 1, rats were exposed via nose-only inhalation for 15 minutes to aerosols of 1) insoluble UO_2; 2) soluble UO_3; 3) a mixture containing by weight 50% UO_2 and 50% UO_3; 4) TaO_2 (a negative control) at concentrations of 500 mg/m^3. A subset of animals was also co-exposed to nasal endotoxin to induce nasal epithelial damage. Rats exposed to inhalation of clean-filtered air served as control. This *"Tank Impact" scenario* has been completed.

During Year 2, serial sacrifices of rats exposed via nose-only inhalation for *6 hours* to aerosols of a mixture containing by weight 50% UO_2 and 50% UO_3 at a dose of 1 mg/m^3 were performed (*"March Through" scenario*). A separate air-only exposed group was used as control. For each group rats will be sacrificed immediately following exposure, and at 30, 180 days post-exposure.

Serial sacrifices of rats exposed via nose-only inhalation for *30 days* to aerosols of a mixture containing by weight 50% UO_2 and 50% UO_3 at a dose of 1 mg/m^3 (*"Clean-Up" scenario*) were also performed in year 2. A separate air-only exposed group was used as a control.

In the re-scope approve at the end of Year 3, we proposed to include additional animals in the *"Clean-up" scenario* exposures to clarify and expand positive findings seen to date. These animals were exposed to long-term, moderate dose of UO_2+UO_3 in Year 3, but a subset of animals was *also* subjected to induced nasal inflammation. Rats were sacrificed immediately after exposure, 14, 30 or 180 days post- exposure. The additional animals allowed us to expand tissue analysis to include uranium levels in blood, kidneys and lungs as well as evaluate neuronal damage an inflammation in spinal cords. Also, brain tissue from these animals will be used for molecular biology analysis of oxidative stress and DNA damage.

Finally, in Year 3 we *re-exposed* animals previously exposed in the "March Through" and "Clean Up" scenarios in Year 2. All re-exposed animals were sacrificed immediately after end of exposure.

Year 4 Scope

The overall scope of year 4 was to complete sacrifices from the second "Clean-Up" scenario and to perform post-analysis of tissue from this exposure and the re-exposure mentioned above.

Quantitation of metals brain tissues will be performed with microbeam Proton Induced X-ray Emission (μ-PIXE). Brain tissues will be examined using immunohistochemistry (IHC) for the persistence of tyrosine hydroxylase - containing neurons. Neuroinflammation will be monitored by GFAP (glial fibrillary acidic protein) or Iba-1 (a marker for microglia) -IHC. A marker of neuronal

degeneration (Fluoro-Jade) will also be examined in sections from brains showing uranium deposition. If data from any of these categories indicates that there is neurodegeneration, specific brain areas such as the substantia nigra (SN) and anatomically-linked areas will be studied in greater detail using IHC directed against markers for neuroinflammation and neuronal damage.

We propose examining the spinal cords in the exposed animals for markers of damage associated with amyotrophic lateral sclerosis, a neurodegenerative disease recently noted to occur at increased prevalence in Gulf War veterans. We will count large spinal motor neurons (>25 micron) in cresyl violet stained sections of cervical and lumbar spinal cords from animals in all conditions in this exposure, in addition to examining microglia.

Snap-frozen brain tissue will be used chiefly to increase our understanding of mechanisms underlying toxicity by looking at early signs of DNA damage and repair, as well as markers of oxidative stress likely to accompany uranium entry into the CNS. These studies will be done in collaboration with Fernando Cardozo-Pelaez, Ph.D., at the University of Montana who has developed methodologies and published in this area on metal neurotoxicity.

We propose to look at U uptake in kidneys to explore the gender difference seen with the high-dose exposures. Females were more sensitive to lethal effects at the high dose exposures, and to date more females have died in the lower-dose exposures as well. Because of the observation of increased sensitivity in the females, we would like to determine, using more sensitive analytical methods for Uranium in organ tissues (ICP-MS), whether we see differential uptake in females that could underlie the increased sensitivity to kidney toxicity.

One kidney and one lung from each sacrificed animal will undergo histopathological examination. Data from the kidney analyses will allow us a measure of potential nephrotoxicity and will afford a comparison of inhalation data with nephrotoxic effects arising from the study of DU implants. Data from lung will afford an assessment of any pulmonary damage resulting from the inhalation exposures.

Progress on Year Four Scope

The following section is organized by task identified in the original scope of work documented above. The relevant section of the scope, quoted and in bold italics, begins each description of work.

A. "Clean-up Scenario": Long duration, moderate dose, with or without endotoxin

TASK 1: ***Completing endotoxin exposure in Clean-up Scenario originally proposed and replacing the Maintenance Scenario with additional animals in the "Clean-up Scenario" exposures to clarify and expand positive findings seen to date.***

NOTE: Animals described under "Clean-Up Scenario" in this report supplement previously reported exposure to the same dose and duration of uranium inhalation.

Methods:

Inhalation exposures and initial sacrifices (0, 14 and 30 days post-exposure) were performed in Year 3 and detailed methods have been reported previously. A summary of the methods is described below.

Animals

A total of 139 (68 male and 71 female) Fischer 344 rats, 9-10 weeks old, (Harlan Sprague Dawley, Indianapolis, IN) were used. The rats were randomized by weight into exposure groups as outlined in Table 1.

Table 1. Experimental groups for "Clean-up Scenario" (1 mg/m^3 UO_2+UO_3) 30day exposure

SURVIVAL TIME	**0 days[a]**		**14 days[b]**		**30 days[b]**		**180 days[c]**	
Condition	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*
UO_2+UO_3	6	6	3	3	3	3	4	5
UO_2+UO_3 + Endotoxin	6	6	3	3	3	3	3	5
Air	6	6	3	3	3	3	4	4
Air + Endotoxin	6	6	3	3	3	3	4	4

M=male, F=female. [a] 3 rats per group and gender was used for histology and 3 rats per group and gender was used for PIXE analysis and molecular biology. [b] all rats used for PIXE analysis and molecular biology. [c] All rats used for histology.

Endotoxin Instillation

To induce inflammation in the nasal mucosa, two groups of rats (Table 1) were intranasally instilled with endotoxin (Sigma Chemical Co., St. Louis, MO,

Lipopolysaccharide from Pseudomonas aerugenosa Serotype 10, 1 mg/ml) following the procedures of Harkema and Hotchkiss (1991). Rats were anesthetized by halothane inhalation, removed in a light plane of anesthesia, and instilled with endotoxin (200 µg total) by placing 2 - 50 µl drops in each nostril. Endotoxin instillations were made weekly (every Saturday), 48 hours before start of each week's 5-day exposure session (Monday-Friday) to uranium aerosols or air.

Generation of UO_2 + UO_3 aerosols

Aerosol of the UO2 + UO3 mixture was generated using a Wright Dust Feeder (BGI, Inc,). Aerosols were diluted with clean, filtered air and passed through a cyclone to remove the fraction of aerosol larger than approximately 5 microns. The aerosol was then fed into a 96-port nose-only exposure system that was operated at a flow rate of approximately 30 L/min.

The measured UO_2 + UO_3 concentration in the aerosol was 1.02 ± 0.04 mg/m^3, size distribution was 1.74 ± 0.16 µm with a sigma-g of 1.57 ± 0.12. Target concentration was 1 mg/ m^3. PIXE analysis of filters from test ports in the incubation chambers confirmed only uranium to be present in each filter. Minimum detection limits for other elements were ~ 0.1 µg/g.

Inhalation exposures

Before exposure, rats were conditioned to nose-only restraint tubes for at least two periods. Rats were exposed nose-only for 30 days (6 h/day, 5 days/week for 6 weeks) to 1.0 mg/m^3 of UO_2 + UO_3 mixture or to clean-filtered air only. See Table 1 for details.

Animal sacrifices and tissue collection

Three male and three female animals per exposed group were sacrificed and perfused with saline at each of the following time-points: 0-4h post-exposure (0 day), 14 days or 30 days post-exposure. A summary of retrieved tissues and planned analysis is outlined in Table 2. After CO_2 inhalation, 2-4ml blood were collected from the heart and frozen at –20°C. Left kidney was tied off, removed, weighed and frozen at –20°C. After intracardial saline perfusion, the brain was removed and weighed. One brain hemisphere was frozen in dry-ice-cooled isopentane at –42°C, and the other hemisphere was frozen in liquid nitrogen. Brain tissue was transferred to –80°C for long-term storage. Cervical and lumbar spinal cord regions were dissected, post-fixed in 4% paraformaldehyde and then transferred to 20% sucrose for storage. Thoracic spinal cord was dissected, frozen in liquid nitrogen and stored at –80°C. The nose, with skin and lower jaw removed was fixed in 4% paraformaldehyde. The left and right lungs were weighed. The left lung was perfused with 4% paraformaldehyde and the right was frozen at –20°C. The larynx, trachea and bronchial lymph node were fixed in 4% paraformaldehyde. The right kidney was weighed and fixed in 4% paraformaldehyde. The carcass was weighed and frozen for chemical analysis.

Three male and three female animals from each exposed group and 2 male and 2 female unexposed animals were perfused with saline followed by 4% paraformaldehyde on the last day of exposure (0 day). Brain and spinal cord were dissected, post-fixed in 4% paraformaledhyde for 24h and then transferred to 20% sucrose for storage. Non-CNS tissue was collected exactly as described above for saline-only perufsed animals.

Table 2. Tissue retrieval and planned analysis for moderate dose (1 mg/m^3 UO_2+UO_3) 30day exposure

Tissue	Preservative	Analysis (Facility)
Saline only perfused animals (0, 14 and 30 days post-exposure)		
1/2 Brain	Freeze –42^{o}C isopentane	Histology (UNM), Uranium uptake (LLNL)
1/2 Brain	Freeze liquid nitrogen	Neurochemistry (U Montana)
Spinal cord (cervical and lumbar)	4% PFA 24 h	Histology (UNM)
Spinal cord (thoracic)	Freeze liquid nitrogen	Neurochemistry (U Montana)
Saline+paraform perfused animals (0 and 180 days post-exposure)		
Brain	4% PFA 24 h	Histology (UNM)
Spinal cord (cervical and lumbar)	4% PFA 24 h	Histology (UNM)
All animals (0, 14, 30 and 180 days post-exposure)		
Blood (heart)	Freeze –20^{o}C	Uranium content (U Santa Cruz)
Nose	4% Paraform.	Histology (LRRI)
Lung - left	4% Paraform	Histology (LRRI)
Lung – right	Freeze –20^{o}C	Uranium content (U Santa Cruz)
Larynx	4% Paraform	Histology (LRRI)
Trachea	4% Paraform	Histology (LRRI)
LN, Bronchial	4% Paraform	Histology (LRRI)
Kidney – left	4%Paraform	Histology (LRRI)
Kidney – right	Freeze –20^{o}C	Uranium content (U Santa Cruz)
Carcass	Freeze –20^{o}C	Uranium content (LLNL)

Results:

Exposure and sacrifices

Animal exposures as well as sacrifices at 0, 14 and 30 days post-exposure were completed in Year 3. The last sacrifice (180 days post-exposure) was performed in Year 4.

Body and brain weights

There was no significant difference in brain or bodyweight between the experimental groups.

TASK 2: ***Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (-PIXE)***

Methods:

Tissue sectioning and preparation
Frozen tissue sections were cut at 10 µm using a Hacker-Bright cryostat. Three levels of sagittal sections (2.9, 1.5 and 0.9 mm lateral of midline) containing the brain regions of interest were sampled. One section from each level was mounted on a nylon foil and freeze-dried for PIXE analysis. One section from the same level was stained with hematoxylin and eosin, digitally scanned, and regions for PIXE analysis were outlined to ensure beam localization within correct brain regions (Figure 1). Additional sections from each level were retained for future immunohistochemical analysis.

PIXE measurement of metal content
Uranium concentrations in localized brain regions (glomeruli, mitral cells, olfactory tuberculum, caudate putamen, and substantia nigra) were determined with Proton Induced X-ray Emission (PIXE). PIXE is an x-ray fluorescence technique that uses MeV energy proton beams to interrogate specimens. It provides accurate quantitation, simultaneous multi-element detection and is capable of micron-scale spatial resolution whilst maintaining down to 1 mg/g elemental sensitivity. Regions of interest within the freeze-dried tissue sections were identified visually using stained adjacent serial sections. Regions of interest were irradiated with 3 MeV proton microbeams for doses of up to 15 micro coulombs. Beam spot sizes were typically between 0.3x0.3 and 0.5x0.5 mm. X-ray yields for uranium were monitored using an energy dispersive x-ray detector. Yields were converted to quantitative concentrations using thin film standards of uranium of known thickness to determine detector efficiency. The

system has been tested on certified standards and has quantitative accuracy of better than 95% for analysis of metals in biological matrices.

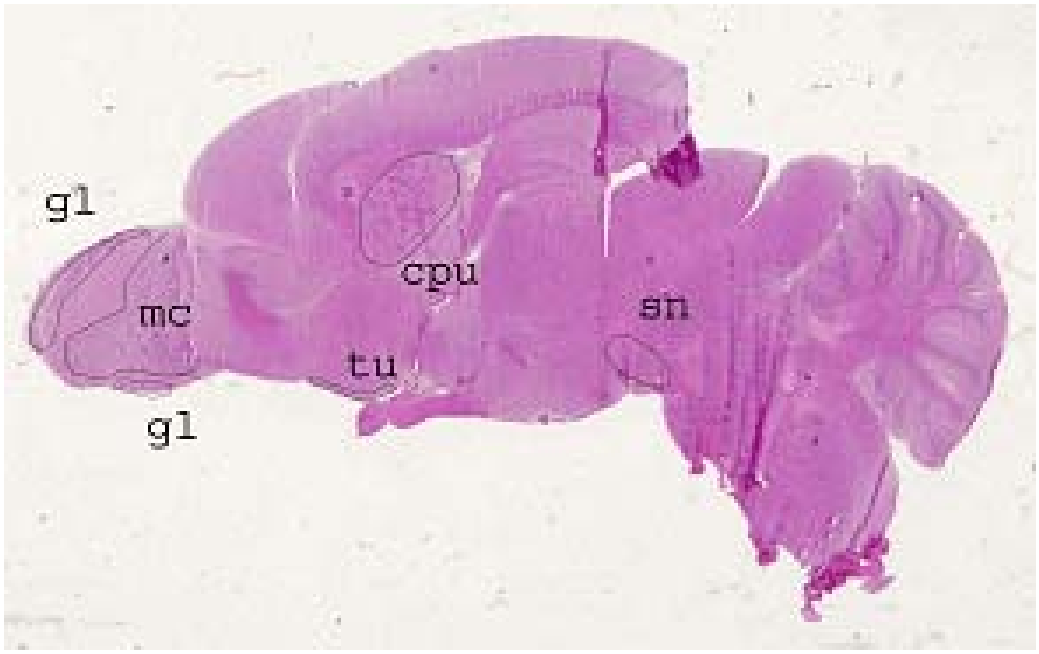

Figure 1. Hematoxylin and eosin stained sagittal brain section with anatomic structures of interest outlined where: gl=glomeruli; mc=mitral cells; tu=tuberculum; cpu=caudate putamen; sn=substantia nigra.

Results:

At the 0 day post-exposure time point, one female animal exposed to UO_2+UO_3 displayed a uranium concentration of 3.1 ± 2.0 mg/kg in the glomeruli. A second female rat exposed to UO_2+UO_3+endotoxin displayed a uranium concentration of 3.0 ± 2.1 mg/kg in the mitral cell layer. The average minimum detection limit was 2.7 mg/kg. The ratio of animals with uranium uptake and total number of exposed animals is outlined in Table 3. Due to the low concentration observed in those animals with detectable uranium, the low frequency of detection, and our previous information on the rate of decrease in other transported brain metals with time and deeper tissue transport, later time-points (14 and 30 days post-exposure) were not analyzed for uranium content.

Table 3 Number of rats with detectable uranium uptake in different brain structures.

	UO_2+UO_3		UO_2+UO_3 + Endotoxin	
Structure	*Male*	*Female*	*Male*	*Female*
Glomeruli	0/6	**1/6**	0/6	0/6
Mitral cells	0/6	0/6	0/6	**1/6**
Olfactory tuberculum	0/6	0/6	0/6	0/6
Caudate putamen	0/6	0/6	0/6	0/6
Globus pallidus	0/6	0/6	0/6	0/6
Substantia nigra	0/6	0/6	0/6	0/6

TASK 3: ***Brain tissues will be examined using immunohistochemistry (IHC) for tyrosine hydroxylase, neuroinflammation and neuronal degeneration.***

Methods

Olfactory bulbs from paraformaldehyde-fixed brain retrieved 0 or 180 days post-exposure were cryosectioned coronally at 10 μm thickness through three levels of the olfactory bulb.

One slide form each animal (containing 3 sections from the olfactory bulb) was stained for immunoreactivity against glial fibrillary acidic protein (GFAP) using a 1:200 dilution of the primary antibody (Jackson Immunoresearch). A second slide was stained for tyrosine hydroxylase (TH) immunoreactivity (1:200 dilution of primary antibody from Pel-Freez, AR) and a third slide was stained for Iba-1 immunoreactivity (1:1000 dilution, Waco Chemicals, VA). Cy3-conjugated secondary antibodies were used for all stains. Finally, a fourth slide was stained with Fluoro-Jade C according to manufacturer's guidelines (Schmued et al. 2005).

Digital photographs of stained sections were captured using an Olympus fluorescent microscope and camera. Intensity of GFAP and TH immunoreactivity in glomeruli was measured using ImageTool software. Intensity of Iba-1 staining in the middle part of the olfactory bulb was measured in a similar fashion. Fluoro-Jade C staining was manually inspected by two observers, quantification is in progress. Data below is presented as mean staining intensity after subtraction of background intensity.

Results

Glial Fibrillary acidic protein immunoreactivity

Glial fibrillary acidic protein (GFAP) was used as a marker for astrogliosis, a sign of neuroinflammation (Fig 2.). Preliminary statistical analysis has been performed. There was no significant difference between male and female rats and data was therefore pooled for both genders. Separate Two-factor ANOVA were perfomed on tissue harvested 0 and 180 days post-exposure since tissue from the two timeponts was stained on two different occasions. Preliminary findings show that uranium inhalation alone, but not endotoxin exposure alone, increase relative intensity of glial fibrillary acidic protein (GFAP) in olfactory glomeruli 180 days post-exposure ($p < 0.05$, $n = 8$ per group). There was no significant effect of uranium alone, endotoxin alone and no uranium and endotoxin interaction in tissue harvested 0 days post-exposure ($p>0.05$, $n=8$ per group).

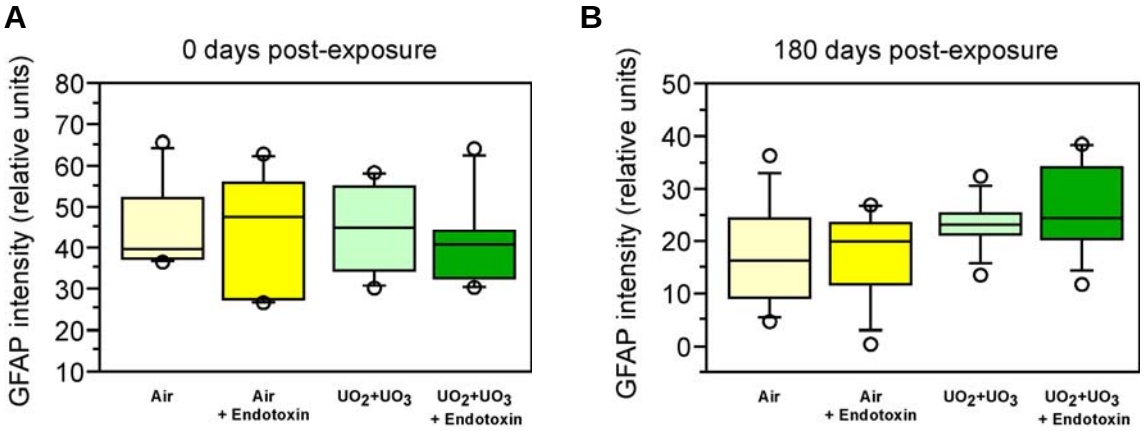

Fig 2. Box plots of relative GFAP immunoreactivity in olfactory glomeruli 0 days (A) or 180 days (B) post-exposure

Iba-1 immunoreactivity

Iba-1 immunohistochemistry was used to visualize microglia as a sign of neuroinflammation. Analysis of iba-1 intensity in the middle part of olfactory bulb did not significantly differ between experimental groups at 0 or 180 days post-exposure. Analysis of Iba-1 intensity in olfactory glomeruli is currently ongoing.

Tyrosine hydroxylase immunoreactivity

Antisera against tyrosine hydroxylase (TH) was used to visualize dopaminergic neurons (Fig 3.). Preliminary statistical Two-factor ANOVA was performed on 0 and 180 day post-exposure tissue and data from male and female rats was pooled. Both uranium exposure alone and endotoxin exposure alone increased

the relative TH intensity in olfactory glomeruli 180 days post-exposure ($p < 0.05$, $n = 8$ per group), but there was no uranium and endotoxin interaction. At 0 days post-exposure there was no statistically significant effect of uranium alone, endotoxin alone and no uranium and endotoxin interaction ($p > 0.05$, $n = 8$ per group).

A **B** **Fig 3.** Box plots of relative TH immunoreactivity in olfactory glomeruli 0 days (A) or 180 days (B) post-exposure

Fluoro-Jade C staining

Fluoro-Jade C was used to visualize dead or dying neurons (Schmued et al. 2005). Preliminary inspection of olfactory bulbs 0 and 180 days post-exposure showed very few stained neuronal processes and almost no stained cell bodies. These preliminary results indicate that there is no major neuronal death in the olfactory bulbs at either timepoint. Quantification of Fluoro-Jade C intensity is ongoing.

TASK 4: *Examining the spinal cords in the exposed animals for markers of damage associated with amyotrophic lateral sclerosis, a neurodegenerative disease recently noted to occur at increased prevalence in Gulf War veterans.*

Methods

Spinal cords were cryoprotected in 20% sucrose and 0.01% sodium azide in PBS. Cervical and lumbar enlargement of the cord was cryosectioned transversely at 10 µm thickness. Sections were collected at regular intervals through the structures. One slide per animal (9 sections per slide, each section 200 µm apart) was stained for Cresyl Violet for general morphology. The total number of large spinal motor neurons (>25 µm in diameter) in the ventral horn was quantified according to standard procedures (Klivieny et al. 1999, Karlsson et al. 2004).

Results

To date, spinal cords from rats sacrificed 180 days post-exposure have been analyzed. For both cervical and lumbar spinal cord, preliminary statistical Two-factor ANOVA analysis did not show a significant effect of uranium exposure alone or endotoxin alone and there was no uranium and endotoxin interaction ($p<0.05$).

Ongoing analysis: Analysis of 180 day post-exposure cervical and lumbar spinal cords immunohistochemically stained for GFAP is in progress. Immunoreactivity against microglia and visualization of damaged neurons using FluoroJade C will

be performed in January 2006. Spinal cords harvested 0 days post-exposure will be analyzed using the same methods as mentioned above.

TASK 5: ***Kidney and lung pathology: Clean-up Scenario – Naïve and Reexposed Animals.***

O day sacrifice, Re-exposed Animals. Nephropathy severity was similar across all exposure conditions, indicating no apparent effect of inhalation of UO_2 + UO_3 on the kidney under these conditions. Alveolar macrophage hyperplasia was greatest in animals exposed to UO_2 + UO_3 originally for 30 days and then re-exposed for an additional 30 days. These animals also showed the greatest concentration of visible particles in alveolar macrophages. Visible particles were observed in alveolar macrophages in nearly all groups of animals-exposed to UO_2 + UO_3 for 30 days, regardless of whether the reexposure was to U or air. The exception to this was in animals co-exposed to endotoxin both initially and during reexposure, suggesting some protective effect of repeated inflammation on lung deposition. If the initial exposure was for only 1 day, retention of particles was uniformly observed immediately following reexposure.

0 day sacrifice, Naïve Animals. Again, no indication of kidney pathology was observed following these exposure conditions. Alveolar macrophage hyperplasia, rated as mild to moderate, was observed in all animals exposed for 30 days to UO_2 + UO_3, with all but one animal showing visible inclusion of particles in the macrophages. Air exposed animals, especially when co-exposed to endotoxin, also showed alveolar macrophage hyperplasia, although not as severe and with no visible particle inclusions. The presence of septal fibrosis and interstitial inflammation was associated with endotoxin instillation regardless of U exposure.

14 day sacrifice, Naïve Animals. Two of three U-exposed male rats, not exposed to endotoxin, at this survival time showed a few, scattered foci of tubular regeneration with thickened basement membrane and karyomegaly. Endotoxin-associated septal fibrosis was still apparent, although interstitial inflammation had resolved. In all U-exposed animals, particles were still visible, and alveolar macrophage hyperplasia was still present at the mild-to moderate level.

30 day sacrifice, Naïve Animals. At this time point no relationship between exposure and kidney pathology was observed. Fibrosis associated with endotoxin exposure continued to resolve. U-associated alveolar macrophage hyperplasia and inclusion of particles remained apparent in nearly all animals at nearly the same severity previously observed.

180 day sacrifice, Naïve Animals. Mild nephropathy of a lesser degree than observed in the 14-day U-exposed males was observed in some animals in all groups at this longer survival time, suggesting a relationship more to age than exposure. Alveolar macrophage hyperplasia and particle inclusions were no longer solely associated with U-exposure at this time-point. Although one animal in each of the U-exposed conditions showed retention of particles, animals in the air-exposed groups also showed some visible particles in macrophages, and minimal hyperplasia as well. The severity of hyperplasia was modestly greater in animals who had been exposed to U.

E. "Re-exposure" scenario

TASK 1: *Re-expose animals previously exposed and held for long-term survival.*

The exposures and sacrifices were completed in Year 3, but brief methods are included again in this Year 4 report for clarity.

Methods:

Animals

A total of 41 rats from the 2003 exposures, originally intended as a 360-day post-exposure analysis group, were selected for re-exposure. Animals were assigned to UO_3+UO_3+endotoxin or air+endotoxin re-exposure groups as outlined in Table 4. Between the first exposure and the re-exposure, animals were housed at standard conditions and weighed monthly.

Table 4. Re-exposure (1 mg U/m^3) of previously exposed (1 mg U/m^3) rats

PREVIOUS EXPOSURE		**RE-EXPOSURE**		**0 day sacrifice**
Condition	*Duration*	*Condition*	*Duration*	*N*
Air	30 day	UO_2+UO_3 + Endotoxin	30 day	4
		Air + Endotoxin	30 day	4
UO_2+UO_3	30 day	UO_2+UO_3 + Endotoxin	30 day	6
		Air + Endotoxin	30 day	4
UO_2+UO_3	1 day	UO_2+UO_3 + Endotoxin	30 day	7
		Air + Endotoxin	30 day	4
UO_2+UO_3 + Endotoxin	1 day	UO_2+UO_3 + Endotoxin	30 day	8
		Air + Endotoxin	30 day	4
Air +Endotoxin	1 day	No exposure	N/A	12

Previous exposure date: 10/1/2003 (1 day exposed animals), 8/25-10/6/2003 (30 day exposed)

Endotoxin instillation and inhalation exposures.

Procedures for endotoxin instillation, animal conditioning and exposure atmosphere generation were as described above for "Clean-up scenario". Briefly, rats were re-exposed to 1.0 mg/m^3 of UO_2+UO_3 mixture or to clean-filtered air for 30 days (6 h/day, 5 days/week for 6 weeks). All re-exposed rats received weekly endotoxin instillations 48 h prior to the weekly exposure session.

Animal sacrifices and tissue collection

All rats were sacrificed at day of completed exposure (0-6h after exposure) and were perfused with saline. Euthanasia and tissue retrieval was identical to procedures described for saline-only perfused rats in "Clean-up scenario" above.

TASK 2: ***Quantitation of metals in nose and brain tissues will be performed with Atomic Absorption Spectrophotometry (AAS) and microbeam Proton Induced X-ray Emission (PIXE)***

Methods: See "Clean-up" exposure scenario above.

Results:
In the Year 3 report we only had analyzed a subset of the re-exposed animals. The PIXE evaluation of brain metal content is now complete and summarized in Table 5. One male rat initially exposed to UO_2+UO_3 for 30 days exhibited a glomerular uranium concentration of 3.8 ± 2.2 mg/kg. One male rat initially exposed to 1 day (6h) of UO_2+UO_3 showed a glomerular uptake of 3.3 ± 2.5 mg/kg uranium. Finally, one female initially exposed to endotoxin plus 1 day (6h) of UO_2+UO_3 showed a glomerular uptake of 3.1 ± 2.3 mg/kg and uranium levels in the mitral cell layer of 3.1 ± 2.2 mg/kg. The average minimum detection limit for all structures was 2.6 - 2.7 mg/kg.

Table 5. Number of re-exposed rats with brain uranium uptake

1st exposure	Air 30 days		UO_2+UO_3 30 days		UO_2+UO_3 1 day		UO_2+UO_3 +endotoxin 30 days	
2nd exposure	**UO_2+UO_3 + Endotoxin, 30 days**							
	M	*F*	*M*	*F*	*M*	*F*	*M*	*F*
Glomeruli	0/2	0/2	**1/4**	0/2	**1/7**	0/0	0/0	**1/7**
Mitral cells	0/2	0/2	0/4	0/2	0/7	0/0	0/0	**1/7**
Olfactory tuberculum	0/2	0/2	0/4	0/2	0/7	0/0	0/0	0/7
Caudate putamen	0/2	0/2	0/4	0/2	0/7	0/0	0/0	0/7
Globus pallidus	0/2	0/2	0/4	0/2	0/7	0/0	0/0	0/7
Substantia nigra	0/2	0/2	0/4	0/2	0/7	0/0	0/0	0/7

KEY RESEARCH ACCOMPLISHMENTS DURING YEAR 4

Clean-up scenario: *Moderate dose, long-term exposures*

- Completion of the 180 day post-exposure sacrifices.
- Analysis of uranium content in brain 0 days post-exposure. Uranium deposition was only seen in the olfactory bulb, mainly in olfactory glomeruli, and only in a subset of animals (2 of 12 animals).
- Preliminary analysis of glial fibrillary acid protein (GFAP) in olfactory glomeruli. Uranium inhalation increase GFAP immunoreactivity at 180 days, but not 0 days post-exposure
- Preliminary analysis of tyrosine hydroxylase (TH) in olfactory glomeruli. Uranium or endotoxin independently increase TH immunoreactivity in at 180 days, but not 0 days post-exposure
- Preliminary analysis of olfactory bulb microglia. There was no significant differences between experimental groups at 0 or 180 days post-exposure
- Preliminary analysis of Fluoro-Jade stained olfactory bulb. There does not appear to be any significant neuronal death 0 or 180 days post-exposure
- Preliminary quantification of large spinal motor neurons in cervical and lumbar spinal cords from rats sacrificed 180 days post-exposure did not reveal a significant difference between experimental groups.
- Only at 14 days was any indication of U-related kidney pathology observed, and this was minimal in 2 of 3 males not co-exposed to endotoxin.
- Lasting alveolar macrophage hyperplasia and particle inclusion was observed in all groups exposed to uranium in these conditions.

Re-exposure scenario: *Moderate dose, long-term exposures of moderate dose exposed animals from Year 2 (initialized in Year 3)*

- Analysis of brain uranium deposition 0 days after re-exposure. As described for the “Clean-Up Scenario” above, uranium deposition was only seen in the olfactory bulb, mainly in olfactory glomeruli, and only in a subset of animals (3 of 24 animals).
- Observed nephropathies in these animals were not exposure-related, but rather likely reflected the increased age of these animals.
- Alveolar macrophage hyperplasia and retention of visible particles was greatest in animals exposed to UO_2 + UO_3 originally for 30 days and then re-exposed for an additional 30 days.
- Visible particles were observed in alveolar macrophages in nearly all groups of animals-exposed to UO_2 + UO_3 for 30 days, regardless of whether the reexposure was to U or air. The exception to this was in animals co-exposed to endotoxin both initially and during reexposure, suggesting some protective effect of repeated inflammation on lung deposition.

REPORTABLE OUTCOMES

A poster with data from the acute uranium exposures was presented at “Society for Neuroscience Annual Meeting” in Washington in October 2005 (Karlsson et al. 2005).. The abstract is attached as Appendix 1.

CONCLUSIONS

To date, PIXE analysis of brain metal content has been performed for all exposure scenarios. For each exposure, only a fraction of animals show detectable uranium levels in the brain. The only brain regions showing uranium deposition were the olfactory glomeruli and the mitral cell layer, the first regions receiving neuronal input from the nasal cavity. At the concentrations and durations of these exposures, no detectable uranium has been found in deeper brain structures such as caudate putamen, globus pallidus or substantia nigra. This pattern of metal distribution suggests a direct neuronal transport of uranium from the nasal cavity as opposed to a secondary systemic delivery of uranium to the brain. The low frequency of occurrence suggests the transport is not uniform in the population, but that individual sensitivity exists. The data do not suggest a major effect of gender or prior endotoxin exposure (nasal inflammation) on the risk for brain uranium deposition.

Histochemical analysis of uranium-exposed brains showed an upregulation of tyrosine hydroxylase and glial fibrillary acidic protein in olfactory bulbs from rats analyzed 6 months, but not immediately after, uranium exposure. The data indicate a delayed inflammatory response in the brain, which has been more uniform in exposed animals than detection of uranium deposition. These data suggest that a CNS response can occur even when uranium deposition is not detectable. Ongoing immunohistochemical analyses are in progress in an effort to more fully understand the implications of these results.

Spinal cords from rats sacrificed 180 days after exposure have been analyzed for loss of large motor neurons. Such a neuronal loss is evident in ALS patients and transgenic ALS mice and rats. We did not see a clear motor neuron loss in the uranium-exposed rats in our study. However, neuronal loss is a relatively late pathological feature in ALS and it is possible that histological markers detected at an early stage in ALS (e.g. inflammation, mitochondrial damage) may also bee seen in our uranium-exposed rats. Such early markers are currently being evaluated.

At these exposure conditions, no clear association of exposure to kidney pathology was observed. However, alveolar macrophage hyperplasia and long-term retention of particles was associated with all groups exposed to uranium.

Problems encountered during the year: Our research group has during Year 4 been faced with several major setbacks and obstacles that in part have delayed the histological evaluation tissue. The laboratory moved to a new building in summer 2005 and the former research technician, Antonia Abeyta, left the group to study medicine at the University of New Mexico. A new research

technician, Bernadette Pacheco, was hired in August 2005. the same month, Dr Johnnye Lewis (PI) was involved in a serious riding accident and needed to undergo major surgery. Dr. Lewis had a second emergency surgery in November, and continues to recover. She hopes to be back in the laboratory in January on at least a part-time basis. She has continued to consult with Dr. Karlsson during her medical leave to ensure work continues, although the pace has been slowed.

Dr. Lewis's absence has also caused a delay in the subcontracts for analytical work at UC Santa Cruz and the University of Montana. Those subcontracts resulted from the rescoping, and require transfer of budget from a LLNL subcontract. Transfer of these funds was waiting on completion of the majority of the analytical work at LLNL, which has now been completed. However, Dr. Lewis has not been able to draft the contract modifications and the new scopes of work to complete the process. This should be complete in January. Tissues are stable at those facilities and awaiting subcontract completion to complete analysis.

Despite these setbacks, we have managed to progress with tissue evaluation and data handling at a reasonable speed and we are confident that several manuscripts will be submitted within the next few months. Because of the comparative nature of these studies, we have wanted to complete comparable analyses in all scenarios and time-points before integrating the work into publications. We are now close to that point.

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APPENDIX 1. Abstract presented at Society for Neuroscience Annual Meeting

URANIUM BRAIN DEPOSITION, CNS INFLAMMATION AND EFFECTS ON OLFACTORY DOPAMINE SYSTEM AFTER NOSE-ONLY INHALATION OF URANIUM OXIDE AEROSOLS IN RATS

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Depleted uranium has been suggested as a potential contributor to Gulf War Illness, which presents with diffuse neurological symptoms. Depleted uranium ammunition can be aerosolized at impact and fine uranium particles are deposited in sand and dust. Inhalation of metals such as manganese results in brain deposition in a pattern consistent with direct metal uptake from olfactory neurons and transport to deeper brain structures. To evaluate brain uranium uptake and CNS damage, male and female rats were exposed by nose-only inhalation to 1.0 mg/m3 aerosolized UO2+UO3 (1:1) for 30 days (6h/day, 5 days/week). Particle size was 1.7 m and sigma-g was 1.6. Control rats were exposed to clean-filtered air for the same duration. Nasal endotoxin instillation was performed in a subset of rats to evaluate effects of nasal epithelial damage on metal deposition. Immediately after the exposure, detectable uranium was found in olfactory glomeruli or mitral cell layers in 5 of 42 rats (range 3.1-3.8 g/g). Minimum detection limit was 2.5-2.7 g/g. Gender and endotoxin instillation did not appear to influence brain uranium uptake. There was no detectable uranium in olfactory tuberculum, caudate putamen, globus pallidus or substantia nigra. Histological evaluation of olfactory glomeruli revealed an increase in glial fibrillary acidic protein (GFAP) and tyrosine hydroxylase (TH) immunoreactivity in uranium-exposed groups 180 days but not 4h after exposure. Assessment of neuronal damage and inflammation in other brain regions and spinal cord is ongoing. The results indicate that low-dose, long-duration uranium inhalation lead to limited olfactory bulb metal deposition, with delayed CNS inflammatory response and TH upregulation. The results are relevant to individuals exposed to uranium inhalation, both in military and civilian environments.

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Appendix II.

Final Report on Kidney and Lung Pathology, Clean-up Scenario
Naïve and Reexposed Animals.

Pathology Report:	**Lovelace Respiratory Research Institute**
Project Title:	**Inhalation of Uranium Oxide Aerosols: CNS Deposition, Neurotoxicity and Role in Gulf War Illness**
Principal Investigator:	**Fletcher F. Hahn, DVM, PhD**
Submitted:	**April 5, 2005**
Submitted to:	**Johnnye Lewis, PhD** **University of New Mexico Health Sciences Center**

Pathology of Inhalation of Uranium Oxide Aerosols

C. Exposure for Thirty Days to 1 mg/m^3 (6 hours/day, 5 days/week, 6 weeks)

Purpose

The purpose was to test the hypothesis that inhaled uranium-containing aerosols will result in central nervous system deposition and subsequent neurodegeneration. This protocol stipulates a six-hour a day, 5 day a week nose-only exposure to aerosols of a uranium oxide mixture for 30 days (six-week elapsed time). Some groups of rats exposed were given weekly intranasal endotoxin instillations and other groups were re-exposed after being previously exposed at least 6 months earlier.

Methods and Materials

Animals and Animal Husbandry

1. Animal Purchase and Housing

A total of 136 F344 rats, 9–10 weeks old, equal numbers of males and females (includes 3 spares of each sex for potential culling), were purchased from Harlan Sprague Dawley (Indianapolis, IN). All rats were quarantined for a minimum of 10 days. In addition, 42 rats previously exposed to uranium oxides were entered in the study for re-exposure. The specific rats re-exposed and the specific numbers for the newly purchased rats are listed in Appendix A. All rats were housed 2 to 3 per cage in shoebox cages with hardwood chip bedding. Rats were fed Teklad Certified Rodent Diet (W). Food and water were available *ad libitum*, except during exposure. The animal rooms were maintained at 20–22°C and relative humidity was 30–70% as much as possible. A 12-hour light/dark cycle was maintained with lights on at 0600.

2. Animal Randomization and Identification

The 130 new rats were randomized by weight, using Path-Tox software, into two exposure groups of 33 rats each and two exposure groups of 32 rats each, equal numbers of males and females (Table 1). The specific rats re-exposed and the specific numbers for the newly purchased rats are listed in Appendix A. A total of 6 rats were available for culling. Rats were identified by tail tattoo using an alpha numeric numbering system.

Table 1. Uranium Entry to CNS – 30 Daily Exposures to 1.0 mg/m^3 (6 hours/day, 5 days/week, 6 weeks)

Group	Previously Exposed	UO_2 + UO_3 (mg/m^3)	Endotoxin (LPS 1/wk)	Total Exposed or Re-exposed	Group Identification
Air	No	0	No	16M/16F	K/L Purchased
LPS + Air	No	0	Yes	16M/16F	M/N Purchased
UO_2 + UO_3	No	1.0	No	16M/17F	P/Q Purchased
UO_2 + UO_3 + LPS	No	1.0	Yes	16M/17F	R/S

					Purchased
30 days-UO_2 + UO_3 Re-exposed to UO_2 + UO_3 + LPS	Yes	1.0	Yes	4M/2F	C/D Re-exposure
30 days-UO_2 + UO_3 Re-exposed to LPS only	Yes	0	Yes	2M/2F	C/D Re-exposure
1 day-UO_2 + UO_3 Re-exposed to UO_2 + UO_3 + LPS	Yes	1.0	Yes	4M/4F	G/H Re-exposure
1 day-UO_2 + UO_3 Re-exposed to LPS only	Yes	0	Yes	2M/2F	G/H Re-exposure
1 day-UO_2 + UO_3 + LPS Re-exposed to UO_2 + UO_3 + LPS	Yes	1.0	Yes	4M/4F	I/J Re-exposure
1 day-UO_2 + UO_3 + LPS Re-exposed to LPS only	Yes	0	Yes	2M/2F	I/J Re-exposure
30 days-Air Re-exposed to UO_{2+} UO_3 + LPS	Yes	1.0	Yes	2M/2F	A/B Re-exposure
30 days-Air Re-exposed to LPS only	Yes	0	Yes	2M/2F	A/B Re-exposure
			Total number of UO-exposed rats	92	
			Total number of air-exposed rats	80	
			Total number of LPS-instilled rats	107	

3. Sentinel Animals

Three of the culled rats were designated as sentinel animals and kept on a bottom shelf in the animal room with the study rats. At the 14 day sacrifice, the sentinel rats were sacrificed and a blood sample taken for serology.

4. Animal Selection and Justification for Use

A previous study in this Institute, funded by this project, has shown that inhaled uranium oxides can pass into the brain through the olfactory epithelium in rats. This work indicates that the rat is a good species to test the factors that influence the passage. The inflammation caused by instillation of endotoxin has been shown in a previous study to enhance the passage of uranium oxides into the brain.

5. Animal Conditioning to Exposure Tubes

Before exposure, the rats were conditioned to nose-only restraint tubes for three periods on three

separate days. The first was for about 1 hour, the second for about 3 hours and the third for about 6 hours.

Chemicals

Uranium Oxides – Purchased from CERAC, Inc. P.O. Box 1178
Milwaukee, WI 53201-1178

UO_2 – CAS# 1344-57-6; 50 mesh, 99.8% purity

UO_3 – CAS# 1344-58-7; Powder, 99.8% purity

$UO_2 + UO_3$ = 50%/50% mixture by weight

Endotoxin – Lipopolysaccharide from Pseudomonas aerugenosa Serotype 10, 1 mg/ml was purchased from Sigma Chemical Co., St. Louis, MO

Endotoxin Instillation

Some groups of rats were intranasally instilled with endotoxin to induce inflammation in the nasal mucosa, following the procedures of Harkema and Hotchkiss (1991). Instillations were done weekly, approximately 48 hours before exposure to particulate aerosols or air. The rats were anesthetized with inhalation anesthesia (halothane). The rats were removed in a light plane of anesthesia. Endotoxin (100 µg total) was instilled by placing 50 µl in each nostril. Preparation of the endotoxin is noted in Appendix B.

Inhalation Exposures

6. Preparation of UO Powder

The powders were ball-milled to approximately 5 micra.

7. Exposures and Exposure Atmosphere Characterization

Groups of rats were exposed nose-only for 6 hours per day to a mixture of 50% UO_2/50% UO_3 or to air (control groups). The rats were exposed 5 days a week for 6 weeks for a total of 30 exposure days.

Aerosols were generated from a Wright dust feeder into a 96 port nose-only chamber. Characterization of the atmosphere included determining the aerosol concentration and size distribution. Aerosol samples, collected on Zefluor filters (1.0 micron pore size, 25 mm diameter, Gelman Sciences, Ann Arbor MI), were taken at two locations, one port from each side of the chamber. Samples were taken from the two sampling ports at the start and end of the exposures each day. A nominal flow of 2 LPM was drawn through each filter. Actual flow rates were measured with rotometers. Aerosol concentrations for the sampling periods were determined by dividing the total mass collected on the filter by the airflow through the filter. In addition, the chamber concentration was monitored continuously using a light scatter mass monitor (RAM-S monitor, GCA, Bedford, CT).

The aerosol particle size distributions were determined using a Lovelace multi-jet cascade impactor (InTox Products, Albuquerque, NM). Aerosol samples were collected a minimum of two times during each exposure. The MMAD and geometric standard deviation were calculated using an LRRI computer code. Target particle size of 2 µm MMAD ensured a high deposition in the nose.

8. Environmental Conditions of the Exposure Chamber

Temperature in the chamber was held between 18 and 22°C monitored at 15 minute intervals. Oxygen concentration was monitored at the same time and maintained above 18%.

Post-Exposure Housing

After exposures, animals were housed in special rooms for animals contaminated with radioactivity. Rats scheduled for the 180 day sacrifice were transferred to UNM animal care facilities as soon as animals for the 30 day sacrifice were designated and they were certified as

free of pathogenic viruses by serology testing.

Observations and Measurements

9. Mortality and Morbidity

Observations of the rats being exposed daily were made by personnel loading and unloading the exposure tubes. After completion of the exposure schedule and on weekends, rats were observed twice daily for morbidity and mortality.

10. Body Weight and Clinical Observations

Body weights were obtained within one week of the beginning of exposures for the purpose of randomization. Body weights were obtained the day before exposures began and monthly thereafter. Detailed clinical observations were made at the time of weighing.

11. Gross Necropsy

Sacrificed Rats

1. A specialized examination was performed on rats in all groups at the time of scheduled sacrifice (Table 2).

2. The necropsy procedures were the same for all groups, air exposed or uranium oxide exposed. The organs and tissues sampled and their preservation is shown in Table 3. The rats were sacrificed by intraperitoneal injection of an overdose of Euthasol® and a body weight obtained. At the 14 and 180 day sacrifice blood was obtained from the heart and sera collected for determination of antibodies to typical rat pathogens.

3. The *brain* was removed first, quickly weighed, and immediately quick-frozen for 5 minutes in -40 to -42°C solution of 2-methyl butane and then stored at -80°C. The brains were subsequently serial cryosectioned and microdissected at UNM. Quantitative AAS and PIXE analysis, IHC and qualitative pathology were done on the brain.

Table 2. Sacrifice Times after 30 Days of Exposure

	Sacrifice After End of Exposure				
Group	0 day	14 days	30 days	180 days	Spares
$UO_2 + UO_3$	6M/6F	3M/3F	3M/3F	3M/3F	1M/2F
$UO_2 + UO_3$ + Endotoxin	6M/6F	3M/3F	3M/3F	3M/3F	1M/2F
Control-Air	6M/6F	3M/3F	3M/3F	3M/3F	1M/1F
Endotoxin + Air	6M/6F	3M/3F	3M/3F	3M/3F	1M/1F

Re-expose 30 days-UO_2 + UO_3	4M/2F	0	0	0	0
Re-expose 30 days-UO_2 + UO_3	2M/2F	0	0	0	0
Re-expose 1 day-UO_2 + UO_3	4M/4F	0	0	0	0
Re-expose 1 day-UO_2 + UO_3	2M/2F	0	0	0	0
Re-expose 1 day-UO_2 + UO_3 + Endotoxin	4M/4F	0	0	0	0
Re-expose 1 day-UO_2 + UO_3 + Endotoxin	2M/2F	0	0	0	0
Re-expose 30 days-Air	2M/2F	0	0	0	0
Re-expose 30 days-Air	2M/2F	0	0	0	0
Total Sacrificed	90	24	24	24	10

4. The *nose*, with skin and lower jaw removed, was fixed in 4% paraformaldehyde. Fixative was flushed into the nasal cavity. IHC and qualitative pathology were done on the nose at UNM. The left and right *lungs* were each weighed. The left lung was perfused with 4% paraformaldehyde and the right was frozen. Qualitative pathology was done on the left lung at LRRI and AAS on the right lung. The *larynx, trachea and bronchial lymph node* were fixed in 4% paraformaldehyde for histopathology at LRRI. The left and right *kidneys* were each weighed. The left kidney was fixed in 4% paraformaldehyde and the right was frozen. Histopathology was done at LRRI and AAS analysis. The *carcass* was frozen for chemical analysis. The carcass consisted of the bone and muscle remaining after evisceration and depelting of the body. The lower jaw was included.

Table 3. Organs Sampled and Preservation

Tissue	Weigh	Preservative	Histopathology	Chemistry	Analysis By
Brain	X	Freeze -40°C	X	X	UNM/LLNL
Nose		4% Paraform.	X		UNM
Lung - left	X	4% Paraform	X		LRRI

Lung - right	X	Freeze -40°C		X	To be determined
Larynx		4% Paraform.	X		LRRI
Trachea		4% Paraform.	X		LRRI
LN, Bronchial		4% Paraform.	X		LRRI
Kidney - left	X	4% Paraform.	X		LRRI
Kidney - right	X	Freeze -40°C		X	To be determined
Carcass	X	Freeze -40°C		X	To be determined
Sera (360 days only)		Freeze			Contract Lab

Histopathologic Examination of Selected Tissues at LRRI

Samples of larynx, trachea, lung, bronchial lymph node, and kidney were examined for lesions by light microscopy. Formalin-fixed tissues samples were embedded in paraffin, sectioned at 5 micra, and stained with hematoxylin and eosin.

The microscopic findings in the lung were graded using the criteria show in Appendix Table A and for the kidney, criteria in Appendix Table B. Renal tubular necrosis is sequelae of uranium toxicity. It is characterized by necrosis of the tubular epithelium in tubules of the pars recta portion of the proximal tubules of the kidney. The epithelium is sloughed into the lumen of the tubule. With time, the epithelium regenerates (Barnett and Metcalf, 1949).

A spontaneous ageing renal disease, nephropathy, is characterized by progressive involvement of glomeruli and tubules, with thickening of basement membranes, focal tubular regeneration, mononuclear inflammatory cell infiltrates, glomerular hyalinization and sclerosis, tubular hyaline casts, interstitial fibrosis, mineralization, and cyst formation. Grading was based on greater severity of the lesions comprising the nephropathy and the extent of involvement (Short and Goldstein, 1992).

Results

Aerosol Exposures

The characterization of the exposure aerosols is shown in Table 4. The exposure concentration was 102% of the target concentration. The size, 1.74 µm MMAD, was in the desired range and the size distribution, $\sum g = 1.57$, was reasonably tight.

Table 4. Characterization of Exposure Aerosols

Exposure Material	Mean Concentration[a] (mg/m^3)	Percent of Target	Particle Size Distribution[a] (Sigma-g)	Particle Size[a] (MMAD)
$UO_2 + UO_3$	1.02 ± 0.04 SD	102	1.57 ± 0.10 SD	1.74 µm ± 0.13 SD

[a]Average over 30 days of exposure

Pathology

12. Zero Day Sacrifice of Re-exposed Rats

Thirty eight re-exposed rats were sacrificed 4 hours after the end of the inhalation exposure period. These re-exposed rats had been previously been exposed to $UO_2 + UO_3$ aerosols for 1 day or 30 days and were re-exposed for 30 days (Table 1). The lesion summary is in Table 5 and the individual animal data in Appendix Table C.

Table 5. Histologic Lesion Summary - Re-Exposure Rats

			Kidney	Lung			
Exposure 30 Days	Re-Exposure 30 Days	Number Examined	Nephropathy	Alv. Mac. Hyperplasia	Inflamm. Interstitial	Fibrosis Focal	Alv. Mac. Particles
Air	Air + Endotoxin	2M/2F	1.00[a]	1.50	0.75	0.25	0.00
Air	$UO_2 + UO_3$ + Endotoxin	2M/2F	0.50	1.75	1.00	0.00	1.00
$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin	3M/2F	0.50	2.17	0.50	0.17	1.33
$UO_2 + UO_3$	Air + Endotoxin	1M/2F	0.00	0.33	0.00	1.00	0.67

			Kidney	Lung			
Exposure 1 Day Exposure	Re-Exposure 30 Days	Number Examined	Nephropathy	Alv. Mac. Hyperplasia	Inflamm. Interstitial	Fibrosis Focal	Alv. Mac. Particles
$UO_2 + UO_3$	Air + Endotoxin	2M/2F	0.50	0.25	0.00	0.00	0.00
$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin	4M/4F	0.25	1.13	0.00	0.13	0.63
$UO_2 + UO_3$ + Endotoxin	Air + Endotoxin	2M/2F	0.50	0.00	0.00	0.00	0.00
$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin	3M/3F	0.67	0.33	0.00	0.00	0.00

[a]Lesion score = sum of individual animal severity scores/number in group.

The nephropathy severity is similar in all re-exposure groups indicating that there was no apparent effect of the inhaled $UO_2 + UO_3$ on the kidney. The alveolar macrophage hyperplasia was greatest in the rats that had been exposed for 30 days and then re-exposed for another 30 days. Particles, presumably UO_x, were seen only in those rats exposed to $UO_2 + UO_3$ aerosols. Focal fibrosis, which was minimal, was seen only in those rats instilled with endotoxin. This fibrosis was probably elated to a resolved broncho-interstitial pneumonia that can occur with instilled endotoxin.

13. Early Deaths After 30 Day Exposure

Two rats (Q011 and Q012) died under anesthesia during the instillation of endotoxin. They died 13 days before the end of the 30 day exposure period.

14. Zero Day Sacrifice After 30 Day Exposure

Forty eight rats were sacrificed 4 hours after the end of inhalation exposure as scheduled (individual animal data in Appendix Table D). The lesion summary for the 0 day sacrifice is in Table 6. Alveolar macrophage hyperplasia and particles in those macrophages were related to exposure to $UO_2 + UO_3$ particles, regardless of sex or endotoxin treatment. Alveolar macrophage hyperplasia was present in all of the endotoxin-treated air exposed controls and about one half of the air only controls. The severity was much less than that of the $UO_2 + UO_3$-exposed rats. No particles were found in the air-exposed rats. A minimal chronic interstitial inflammation and a minimal focal septal fibrosis were found in only a few rats that were treated with endotoxin, regardless of particle exposure.

Table 6. Histologic Lesion Summary – 0 days Post Exposure Sacrifice

			Kidney		Lung							
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis		Interstitial Inflammation[a]	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	1/6	0.17	0/6	–	4/6	0.67	0/6	-	0/6	–
	N	F	0/6	–	0/6	–	3/6	0.5	0/6	-	0/6	–
Air	Y	M	0/6	–	0/6	–	6/6	1.7	2/6	0.50	4/6	1.2
	Y	F	0/6	–	0/6	–	6/6	1.2	2/6	0.50	2/6	0.33
$UO_2 + UO_3$	N	M	1/6	0.17	6/6	1.0	6/6	2.2	0/6	–	0/6	–
	N	F	0/6	–	6/6	1.0	6/6	2.2	0/6	–	0/6	–
$UO_2 + UO_3$	Y	M	0/6	–	5/6	0.83	6/6	2.3	4/6	0.67	2/6	0.50
	Y	F	0/6	–	5/6	0.83	6/6	2.0	6/6	1.0	2/6	0.33

[a]Chronic focal interstitial inflammation.

Inc. = # with lesion/# in group; Sev. = total severity score/# in group.

15. Fourteen Day Sacrifice After 30 Day Exposure

Twenty four rats were sacrificed 14 days after the end of inhalation exposure (Table 7). The relationship of histologic changes and exposure was similar in the rats sacrificed at 0 days. The incidence and severity of alveolar macrophage hyperplasia and the presence of particles in the alveolar macrophages were similar. Chronic interstitial inflammation was absent in all but one air control. The incidence of focal septal fibrosis, however, was similar to that seen in the rats sacrificed at 0 days.

Table 7. Histologic Lesion Summary – 14 Days Post Exposure Sacrifice

			Kidney		Lung							
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis		Interstitial Inflammation[a]	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	0/3	–	0/3	–	1/3	0.33	0/3	–	0/3	–
	N	F	0/3	–	0/3	–	0/3	–	0/3	–	1/3	0.33
Air	Y	M	0/3	–	0/3	–	1/3	0.33	3/3	1.0	0/3	–
	Y	F	0/3	–	0/3	–	2/3	0.68	2/3	0.67	0/3	–
$UO_2 + UO_3$	N	M	3/3	1.0	3.3	1.0	3/3	2.3	0/3	–	0/3	–
	N	F	0/3	–	3/3	1.0	3/3	2.0	0/3	–	0/3	–
$UO_2 + UO_3$	Y	M	0/3	–	3/3	1.0	3/3	2.3	3/3	1.0	0/3	–
	Y	F	0/3	–	3/3	1.0	3/3	2.3	1/3	0.33	0/3	–

[a]Chronic focal interstitial inflammation.
Inc. = # with lesion/# in group; Sev. = total severity score/# in group.

16. Thirty Day Sacrifice After 30 Day Exposure

Twenty four rats were sacrificed 30 days after the end of inhalation exposure (Table 8). The relationship of histologic changes and exposure was similar in the rats sacrificed at both 0 and 14 days. The incidence and severity of alveolar macrophage hyperplasia and the presence of particles in the alveolar macrophages were similar. However, chronic interstitial inflammation was absent in all rats. In addition, focal septal fibrosis was present, but with a much lower incidence and severity than that seen in the rats sacrificed at 14 days.

Table 8. Histologic Lesion Summary – 30 Days Post Exposure Sacrifice

			Kidney		Lung							
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis		Interstitial Inflammation[a]	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	0/3	–	0/3	–	3/3	1.0	0/3	–	0/3	–
	N	F	1/3	0.33	0/3	–	2/3	0.67	0/3	–	0/3	–
Air	Y	M	0/3	–	0/3	–	3/3	1.3	1/3	0.33	0/3	–
	Y	F	0/3	–	0/3	–	3/3	1.7	0/3	–	0/3	–
$UO_2 + UO_3$	N	M	2/3	0.67	3/3	1.0	3.3	2.3	0/3	–	0/3	–
	N	F	0/3	–	3/3	1.0	2/3	1.0	0/3	–	0/3	–
$UO_2 + UO_3$	Y	M	0/2	–	2/2	1.0	2/2	2.0	½	0.50	0/2	–
	Y	F	0/3	–	3/3	1.0	3/3	2.0	0/3	–	0/3	–

[a]Chronic focal interstitial inflammation.
Inc. = # with lesion/# in group; Sev. = total severity score/# in group.

17. One Hundred Eighty Day Sacrifice After 30 Day Exposure

Thirty three rats were sacrificed 180 days after the end of inhalation exposure (Table 9). Differences in histologic reactions were very slight among the four exposure groups and between males and females. Particles were present in the alveolar macrophages in one rat in each group, except the endotoxin-treated females exposed to air. In addition, one male in each exposure group had septal fibrosis. Nephropathy was present in one to three rats in each exposure group except for one. The severity was minimal in most cases.

Table 9. Histologic Lesion Summary – 180 Days Post Exposure Sacrifice

			Kidney		Lung							
			Nephropathy		AM Particles		AM Hyperplasia		Septal Fibrosis		Interstitial Inflammation[a]	
Exposure	Endo-toxin	Sex	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.	Inc.	Sev.
Air	N	M	2/4	0.50	¼	0.25	0/4	–	¼	0.25	¼	–
	N	F	2/4	0.75	¼	0.25	¼	0.25	0/4	–	0/4	–
Air	Y	M	¾	0.75	¼	0.25	¼	0.50	¼	0.25	2/4	0.50
	Y	F	0/4	–	0/4	–	0/4	–	0/4	–	0/4	–
$UO_2 + UO_3$	N	M	¾	0.75	¼	0.25	0/4	–	¼	0.25	0/4	–
	N	F	2/5	0.40	1/5	0.40	0/5	–	0/5	–	0/5	–
$UO_2 + UO_3$	Y	M	1/3	0.33	1/3	0.67	1/3	0.33	1/3	0.67	0/3	–
	Y	F	1/5	0.20	1/5	0.21	0/5	–	0/5	–	0/5	–

[a]Chronic focal interstitial inflammation.
Inc. = # with lesion/# in group; Sev. = total severity score/# in group.

Discussion

The reaction to the inhaled $UO_2 + UO_3$ over a 30 day period was mild to moderate alveolar macrophage hyperplasia with a minimal presence of particles in the macrophages. These changes waned slightly from the end of exposure to 30 days after exposure. After 180 days, no difference was noted in the reactions among the exposure groups or between males and females.
At the end of the exposure period alveolar septal fibrosis and chronic interstitial inflammation were present in males and females exposed to endotoxin or to $UO_2 + UO_3$ and endotoxin.
Nephropathy was seen only sporadically in rats before the 180 day sacrifice. Finding the nephropathy at this later time indicates that it is most likely a spontaneous aging lesion, not the result of the renal toxicity of uranium.

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APPENDIX

Table A. Histologic Criteria for Grading Severity of Lung Lesions

Diagnosis	Grade	Criteria
Particle loading of alveolar macrophages (AMs)	–[a]	Essentially no particles in scant cytoplasm
	1	A few black particles scattered in cytoplasm or a few macrophages with abundant particles
	2	Moderate number of particles in cytoplasm (≤10); do not obscure nucleus of macrophage
	3	Many particles (too many to count) in cytoplasm cover the nucleus; slightly enlarged cytoplasm
Alveolar macrophage hyperplasia	–	Few scattered AMs in alveoli; difficult to find
	1	Minimal increase in number of AMs
	2	Mild increase in number of AMs; easily found at high magnification; average 1/alveolus
	3	Moderate increase in number of AMs; easily found at low magnification; several macrophages/ alveolus
Broncho-interstitial pneumonia	–	No inflammation present
	1	A few inflammatory cells infiltrating septa around bronchioles; involves <10% of lung
	2	Infiltrating inflammatory cells involving 10–25% of the lung; may also be in alveoli
	3	Infiltrating inflammatory cells involving 26–50% of the lung; also in alveoli
	4	Infiltrating inflammatory cells involving >50% of lung; also in alveoli
Uremic pneumonia	1	Edema and a few (PMNs) in alveoli; involves <10% of lung
	2	Edema and PMNs in alveoli; Calcium (Ca) present; involves 10–25% of lung
	3	Edema and PMNs in alveoli; Ca present; involves 26–50% of lung
	4	Edema and PMNs in alveoli; Ca present; involves >50% of lung

[a]No abnormalities detected.

Table B. Histologic Criteria for Grading Microscopic Findings in the Kidney

Diagnosis	Grade	Criteria
Tubular Necrosis	–	No tubular necrosis
	1	Few, scattered foci of tubular necrosis; slough of tubular epithelial cells
	2	Mild tubular necrosis in pars recta, sloughing of cells, hyaline casts; involves 5–25% of the proximal tubules
	3	Moderate, tubular necrosis in pars recta, sloughing of cells, hyaline casts; involves 25–50% of the proximal tubules
		Marked, tubular necrosis in the pars recta, sloughing of cells, hyaline casts, mineralization; involves >50% of the proximal tubules
Nephropathy	–	No evidence of nephropathy
	1	Few, scattered foci of tubular regeneration with thickened basement membrane and karyomegaly
	2	Mild tubular regeneration, hyaline casts and cellular infiltrates; involves <25% of the cortical parenchyma
	3	Moderate, tubular, and glomerular fibrosis; involves 25–50% of the cortical parenchyma
	4	Marked, tubular, and glomerular fibrosis; involves >50 % of the cortical parenchyma

Table C1. Individual Animal Histology

#	Ani #	Sex	Exposure 30 Days	Re-Exposure 30 Days	Death Date	Death Type	Days After	Kidney	Lung			
								Nephropathy	AM Hyper	Ch infl Inter	Fibrosis foc	Part /
2	A001	M	Air	$UO_2 + UO_3$ + Endotoxin		sac	0	1	2	0	0	1
2	A002	M	Air	$UO_2 + UO_3$ + Endotoxin		sac	0	1	2	1	0	1
2	A003	M	Air	Air + Endotoxin		sac	0	1	2	1	0	0
2	A004	M	Air	Air + Endotoxin		sac	0	2	2	0	1	0
3	B001	F	Air	$UO_2 + UO_3$ + Endotoxin		sac	0	0	0	1	0	1
3	B002	F	Air	$UO_2 + UO_3$ + Endotoxin		sac	0	0	3	2	0	1
3	B003	F	Air	Air + Endotoxin		sac	0	1	2	1	0	0
3	B004	F	Air	Air + Endotoxin		sac	0	0	0	1	0	0
4	C001	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac	0	1	2	1	0	2
4	C002	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac	0	0	3	1	1	2
4	C003	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac	0	2	2	1	0	1
4	C004	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac	0	0	2	0	0	1
4	C005	M	$UO_2 + UO_3$	Air + Endotoxin		sac	0	0	0	0	2	0
4	C006	M	$UO_2 + UO_3$	Air + Endotoxin	07/26/04	died	8	0	0	0	1	0
5	D001	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac	0	0	1	0	0	1
5	D002	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin	09/03/03	fnd dead	10	4	0	0	0	0
5	D003	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac	0	0	3	0	0	1
5	D004	F	$UO_2 + UO_3$	Air + Endotoxin		sac	0	0	1	0	1	1
5	D005	F	$UO_2 + UO_3$	Air + Endotoxin		sac	0	0	0	0	0	1

Table C2. Individual Animal Histology

Ani #	Sex	Exposure 1 Day Exposure	Re-exposure 30 Days	Death Date	Death Type	Days After	Kidney	Lung			
							Nephropathy	AM Hyper	Ch infl Inter	Fibrosis foc	Par
G001	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		0	1	0	1	
G002	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		0	1	0	0	
G003	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		1	1	0	0	
G004	M	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		0	1	0	0	
G005	M	$UO_2 + UO_3$	Air + Endotoxin		sac		1	0	0	0	
G006	M	$UO_2 + UO_3$	Air + Endotoxin		sac		0	0	0	0	
H001	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		0	1	0	0	
H002	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		0	2	0	0	
H003	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		0	1	0	0	
H004	F	$UO_2 + UO_3$	$UO_2 + UO_3$ + Endotoxin		sac		1	1	0	0	
H005	F	$UO_2 + UO_3$	Air + Endotoxin		sac		1	0	0	0	
H006	F	$UO_2 + UO_3$	Air + Endotoxin		sac		0	1	0	0	
I001	M	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin		sac		1	0	0	0	
I002	M	$UO_2 + UO_3$ + Endotoxin	Vet: Face Mass	07/14/04	euth	287	0	0	0	0	
I003	M	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin		sac		0	1	0	0	
I004	M	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin		sac		0	0	0	0	
I005	M	$UO_2 + UO_3$ + Endotoxin	Air + Endotoxin		sac		0	0	0	0	
I006	M	$UO_2 + UO_3$ + Endotoxin	Air + Endotoxin		sac		1	0	0	0	
J001	F	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin		sac		1	1	0	0	
J002	F	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin	07/25/04	fnd dead	7	1	0	0	0	
J003	F	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin		sac		1	0	0	0	
J004	F	$UO_2 + UO_3$ + Endotoxin	$UO_2 + UO_3$ + Endotoxin		sac		1	0	0	0	
J005	F	$UO_2 + UO_3$ + Endotoxin	Air + Endotoxin		sac		1	0	0	0	
J006	F	$UO_2 + UO_3$ + Endotoxin	Air + Endotoxin		sac		0	0	0	0	

Table D1. Individual Animal Histology

ZERO Day						Kidney	Lung					
Exp #	Ani #	Sex		Type	DPE	Nephropathy	AM Hyper	Alv. Histio	Ch infl Inter	Fibrosis foc	Part /AM	GCL
7538	K001	M	Air / Control	sac	0	0	0	0	0	0	0	0
7538	K004	M	Air / Control	sac	0	0	1	0	0	0	0	0
7538	K005	M	Air / Control	sac	0	0	0	1	0	0	0	0
7538	K009	M	Air / Control	sac	0	0	1	0	0	0	0	0
7538	K015	M	Air / Control	sac	0	0	1	0	0	0	0	0
7538	K016	M	Air / Control	sac	0	1	1	0	0	0	0	0
7539	L001	F	Air / Control	sac	0	0	1	0	0	0	0	0
7539	L002	F	Air / Control	sac	0	0	0	0	0	0	0	0
7539	L003	F	Air / Control	sac	0	0	0	0	0	0	0	0
7539	L004	F	Air / Control	sac	0	0	1	0	0	0	0	0
7539	L007	F	Air / Control	sac	0	0	1	0	0	0	0	0
7539	L015	F	Air / Control	sac	0	0	0	0	0	0	0	0
7540	M004	M	Air / Endotoxin	sac	0	0	2	0	1	0	0	0
7540	M005	M	Air / Endotoxin	sac	0	0	2	0	1	1	0	0
7540	M008	M	Air / Endotoxin	sac	0	0	2	0	0	0	0	0
7540	M012	M	Air / Endotoxin	sac	0	0	2	0	2	0	0	0
7540	M014	M	Air / Endotoxin	sac	0	0	1	0	0	0	0	0
7540	M015	M	Air / Endotoxin	sac	0	0	1	0	3	2	0	0
7541	N006	F	Air / Endotoxin	sac	0	0	1	0	0	0	0	0
7541	N007	F	Air / Endotoxin	sac	0	0	1	0	1	0	0	0
7541	N011	F	Air / Endotoxin	sac	0	0	2	0	0	0	0	0
7541	N012	F	Air / Endotoxin	sac	0	0	1	0	1	1	0	0
7541	N014	F	Air / Endotoxin	sac	0	0	1	0	0	2	0	0
7541	N015	F	Air / Endotoxin	sac	0	0	1	0	0	0	0	0
7542	O005	M	$UO_2 + UO_3$	sac	0	0	3	0	0	0	1	0
7542	O007	M	$UO_2 + UO_3$	sac	0	1	3	0	0	0	1	0
7542	O008	M	$UO_2 + UO_3$	sac	0	0	2	0	0	0	1	0
7542	O010	M	$UO_2 + UO_3$	sac	0	0	1	0	0	0	1	0
7542	O014	M	$UO_2 + UO_3$	sac	0	0	2	0	0	0	1	0
7542	O016	M	$UO_2 + UO_3$	sac	0	0	2	0	0	0	1	0

Table D1. Individual Animal Histology (Concluded)

ZERO Day						Kidney	Lung					
Exp #	Ani #	Sex		Type	DPE	Nephropathy	AM Hyper	Alv. Histio	Ch infl Inter	Fibrosis foc	Part /AM	GCL
7543	P001	F	$UO_2 + UO_3$	sac	0	0	1	0	0	0	1	0
7543	P006	F	$UO_2 + UO_3$	sac	0	0	2	0	0	0	1	0
7543	P008	F	$UO_2 + UO_3$	sac	0	0	2	0	0	0	1	0
7543	P010	F	$UO_2 + UO_3$	sac	0	0	3	0	0	0	1	0
7543	P012	F	$UO_2 + UO_3$	sac	0	0	3	0	0	0	1	0
7543	P013	F	$UO_2 + UO_3$	sac	0	0	2	0	0	0	1	0
7544	Q004	M	$UO_2 + UO_3$ + Endotoxin	sac	0	0	2	0	2	1	1	0
7544	Q005	M	$UO_2 + UO_3$ + Endotoxin	sac	0	0	2	0	0	0	1	0
7544	Q006	M	$UO_2 + UO_3$ + Endotoxin	sac	0	0	3	0	0	1	1	0
7544	Q008	M	$UO_2 + UO_3$ + Endotoxin	sac	0	0	3	0	1	1	1	0
7544	Q010	M	$UO_2 + UO_3$ + Endotoxin	sac	0	0	2	0	0	1	1	0
7544	Q015	M	$UO_2 + UO_3$ + Endotoxin	sac	0	0	2	0	0	0	1	0
7545	R001	F	$UO_2 + UO_3$ + Endotoxin	sac	0	0	3	0	1	1	1	0
7545	R002	F	$UO_2 + UO_3$ + Endotoxin	sac	0	0	3	0	1	1	0	0
7545	R004	F	$UO_2 + UO_3$ + Endotoxin	sac	0	0	2	0	0	1	1	0
7545	R011	F	$UO_2 + UO_3$ + Endotoxin	sac	0	0	1	0	0	1	1	0
7545	R012	F	$UO_2 + UO_3$ + Endotoxin	sac	0	0	2	0	0	1	1	0
7545	R016	F	$UO_2 + UO_3$ + Endotoxin	sac	0	0	1	0	0	1	1	0

Table D2. Individual Animal Histology

14 Day						Kidney	Lung					
o #	Ani #	Sex		Type	DPE	Nephropathy	AM Hyper	Alv. Histio	Ch infl Inter	Fibrosis foc	Part /AM	GC
38	K006	M	Air / Control	sac	14	0	0	1	0	0	0	0
38	K007	M	Air / Control	sac	14	0	1	0	0	0	0	0
38	K014	M	Air / Control	sac	14	0	0	0	0	0	0	0
39	L009	F	Air / Control	sac	14	0	0	0	1	0	0	0
39	L012	F	Air / Control	sac	14	0	0	0	0	0	0	0
39	L013	F	Air / Control	sac	14	0	0	0	0	0	0	0
40	M006	M	Air / Endotoxin	sac	14	0	0	0	0	1	0	0
40	M010	M	Air / Endotoxin	sac	14	0	0	0	0	1	0	0
40	M011	M	Air / Endotoxin	sac	14	0	1	1	0	1	0	0
41	N001	F	Air / Endotoxin	sac	14	0	1	0	0	1	0	0
41	N005	F	Air / Endotoxin	sac	14	0	1	0	0	1	0	0
41	N010	F	Air / Endotoxin	sac	14	0	0	0	0	0	0	0
42	O002	M	$UO_2 + UO_3$	sac	14	1	3	0	0	0	1	0
42	O003	M	$UO_2 + UO_3$	sac	14	1	2	0	0	0	1	0
42	O012	M	$UO_2 + UO_3$	sac	14	1	2	0	0	0	1	0
43	P003	F	$UO_2 + UO_3$	sac	14	0	2	0	0	0	1	0
43	P014	F	$UO_2 + UO_3$	sac	14	0	2	0	0	0	1	0
43	P017	F	$UO_2 + UO_3$	sac	14	0	2	0	0	0	1	0
44	Q001	M	$UO_2 + UO_3$ + Endotoxin	sac	14	0	2	0	0	1	1	0
44	Q003	M	$UO_2 + UO_3$ + Endotoxin	sac	14	0	2	0	0	1	1	0
44	Q009	M	$UO_2 + UO_3$ + Endotoxin	sac	14	0	3	1	0	1	1	0
45	R007	F	$UO_2 + UO_3$ + Endotoxin	sac	14	0	2	0	0	1	1	0
45	R008	F	$UO_2 + UO_3$ + Endotoxin	sac	14	0	2	0	0	0	1	0
45	R009	F	$UO_2 + UO_3$ + Endotoxin	sac	14	0	3	0	0	0	1	0

Table D3. Individual Animal Histology

30 Day						Kidney	Lung					
Exp #	Ani #	Sex		Type	DPE	Nephropathy	AM Hyper	Alv. Histio	Ch infl Inter	Fibrosis foc	Part /AM	GCL
7538	K008	M	Air / Control	sac	30	0	1	1	0	0	0	0
7538	K012	M	Air / Control	sac	30	0	1	0	0	0	0	0
7538	K013	M	Air / Control	sac	30	0	1	0	0	0	0	0
7539	L010	F	Air / Control	sac	30	1	1	0	0	0	0	0
7539	L014	F	Air / Control	sac	30	0	1	0	0	0	0	0
7539	L016	F	Air / Control	sac	30	0	0	0	0	0	0	0
7540	M003	M	Air / Endotoxin	sac	30	0	2	0	0	0	0	0
7540	M009	M	Air / Endotoxin	sac	30	0	1	0	0	1	0	0
7540	M013	M	Air / Endotoxin	sac	30	0	1	0	0	0	0	0
7541	N002	F	Air / Endotoxin	sac	30	0	2	0	0	0	0	0
7541	N008	F	Air / Endotoxin	sac	30	0	2	0	0	0	0	0
7541	N013	F	Air / Endotoxin	sac	30	0	1	0	0	0	0	0
7542	O001	M	$UO_2 + UO_3$	sac	30	0	3	0	0	0	1	0
7542	O004	M	$UO_2 + UO_3$	sac	30	1	3	1	0	0	1	0
7542	O009	M	$UO_2 + UO_3$	sac	30	1	1	0	0	0	1	0
7543	P002	F	$UO_2 + UO_3$	sac	30	0	0	2	0	0	1	0
7543	P009	F	$UO_2 + UO_3$	sac	30	0	1	0	0	0	1	0
7543	P016	F	$UO_2 + UO_3$	sac	30	0	2	0	0	0	1	0
7544	Q002	M	$UO_2 + UO_3$ + Endotoxin	sac	30	0	2	0	0	1	1	0
7544	Q007	M	$UO_2 + UO_3$ + Endotoxin	sac	30	0	2	0	0	0	1	0
7545	R005	F	$UO_2 + UO_3$ + Endotoxin	sac	30	0	2	0	0	0	1	0
7545	R006	F	$UO_2 + UO_3$ + Endotoxin	sac	31	0	2	0	0	0	1	0
7545	R015	F	$UO_2 + UO_3$ + Endotoxin	sac	30	0	2	0	0	0	1	0

Table D4. Individual Animal Histology

180 Day						Kidney	Lung					
Exp #	Ani #	Sex		Type	DPE	Nephropathy	AM Hyper	Alv. Histio	Ch infl Inter	Fibrosis foc	Part /AM	GCL
7538	K002	M	Air / Control	sac	180	0	0	0	0	0	0	0
7538	K003	M	Air / Control	sac	180	1	0	0	0	0	0	0
7538	K010	M	Air / Control	sac	180	0	0	0	0	0	1	0
7538	K011	M	Air / Control	sac	180	1	0	0	0	1	0	0
7539	L005	F	Air / Control	sac	180	2	1	0	0	0	1	0
7539	L006	F	Air / Control	sac	180	0	0	0	0	0	0	0
7539	L008	F	Air / Control	sac	180	0	0	0	0	0	0	0
7539	L011	F	Air / Control	sac	180	1	0	0	0	0	0	0
7540	M001	M	Air / Endotoxin	sac	180	1	0	0	1	1	1	0
7540	M002	M	Air / Endotoxin	sac	180	1	0	0	0	0	0	0
7540	M007	M	Air / Endotoxin	sac	180	0	2	0	1	0	0	0
7540	M016	M	Air / Endotoxin	sac	180	1	0	0	0	0	0	1
7541	N003	F	Air / Endotoxin	sac	180	0	0	0	0	0	0	0
7541	N004	F	Air / Endotoxin	sac	180	0	0	0	0	0	0	0
7541	N009	F	Air / Endotoxin	sac	180	0	0	0	0	0	0	0
7541	N016	F	Air / Endotoxin	sac	180	0	0	0	0	0	0	2
7542	O006	M	$UO_2 + UO_3$	sac	180	1	0	0	0	1	0	0
7542	O011	M	$UO_2 + UO_3$	sac	180	0	0	0	0	0	0	0
7542	O013	M	$UO_2 + UO_3$	sac	180	1	0	0	0	0	0	2
7542	O015	M	$UO_2 + UO_3$	sac	180	1	0	0	0	0	1	0
7543	P004	F	$UO_2 + UO_3$	sac	180	1	0	0	0	0	0	0
7543	P005	F	$UO_2 + UO_3$	sac	180	1	0	0	0	0	2	0
7543	P007	F	$UO_2 + UO_3$	sac	180	0	0	0	0	0	0	0
7543	P011	F	$UO_2 + UO_3$	sac	180	0	0	0	0	0	0	0
7543	P015	F	$UO_2 + UO_3$	sac	180	0	0	0	0	0	0	0
7544	Q013	M	$UO_2 + UO_3$ + Endotoxin	sac	180	0	0	0	0	2	0	0
7544	Q014	M	$UO_2 + UO_3$ + Endotoxin	sac	180	0	1	0	0	0	2	0
7544	Q016	M	$UO_2 + UO_3$ + Endotoxin	sac	180	1	0	0	0	0	0	0
7545	R003	F	$UO_2 + UO_3$ + Endotoxin	sac	180	1	0	0	0	0	1	0
7545	R010	F	$UO_2 + UO_3$ + Endotoxin	sac	180	0	0	0	0	0	0	0

7545	R013	F	UO_2 + UO_3 + Endotoxin	sac	180	0	0	0	0	0	0	0
7545	R014	F	UO_2 + UO_3 + Endotoxin	sac	180	0	0	0	0	0	0	0
7545	R017	F	UO_2 + UO_3 + Endotoxin	sac	180	0	0	0	0	0	0	0